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FINAL REPORT

TARGET ORIENTED DRUGS AGAINST LEISHMANIA

URI ZEHAVI, Ph.D.

and

JOSEPH EL-ON, Ph.D.

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick Maryland 21701-5012

Contract No. DAMD17-79-G-9452 and DAMD17-80-G-9474

Faculty of Agriculture
The Hebrew University of Jerusalem
P.O. Box 12, Rehovot 76-100, Israel

Approved for public release; distribution unlimited

The findings in this report are not to be construed as
an official Department of the Army position unless so
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) 1. Excreted Factor (EF) is a carbohydrate-rich material released by different strains of Leishmania during growth. It has antigenic properties similar to those of the intact parasite and plays a role in the infective process. 2. Isolation and purification of EF is necessary for:		

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- (a) study of its biological function
 - (b) the use of EF for diagnostic purposes
 - (c) the use of EF in immunization experiments
 - (d) the study of the biosynthesis of EF
 - (e) the preparation of inhibitors of particular biosynthetic steps of EF.
3. Purification of EF was achieved by classical extraction and chromatography procedures followed by affinity chromatography. The purified preparations were characterized stressing the carbohydrate moiety in EF which contains a high proportion of galactose. The results are instrumental in more advanced physical, chemical and immunological studies.
 4. We have developed a radioimmunoassay for leishmaniasis utilizing purified EF. The assay can distinguish between Leishmania strains and once further developed, should prove most valuable for the diagnosis of the disease.
 5. EF plays a role in the infective process of Leishmania. We have now shown that surface carbohydrate, related to EF, plays a role in the initial attachment of Leishmania promastigotes to macrophages -- a stage that is a prelude to their engulfment by the macrophages followed by multiplication in the cells.

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1. Introduction

Excreted factor (EF) is a carbohydrate-rich protein excreted by different strains of Leishmania. Our first annual report has demonstrated the presence of very high galactose content in EF, a property that could be shared also by the surface carbohydrates of the parasite. EF has immunological properties similar to those of the intact parasite and may play a role in the infective process. After it was shown to be species-specific, EF became the basis for serotyping Leishmania strains, and is a valuable tool in diagnostic, demographic and ecological studies of the disease^{1,2}. The diagnostic methodology, however, is generally insufficient and the development of a reliable radioimmunoassay (RIA) seems most valuable.

2. Purification and Characterization of EF

EF is produced in relatively small quantities by Leishmania promastigotes in culture. Purified EF was required for (a) RIA development, (b) attachment studies and (c) chemical and physical analysis.

EFs from both L. tropica (L137) and L. donovani (L52) were previously partially purified by physical and chemical methods including ammonium sulphate and acid precipitation, as well as chromatography on Sephadex columns^{3,4,5,6}.

In all these procedures, some of them rather harsh, substances originating from the growth medium were found to contaminate the EF preparations. This level of purification was not satisfactory for either analytical purposes nor for studying the biological function of EF.

The present work includes two new approaches to this problem, established by following two leads: (a) EF from L. donovani inhibited β -galactosidase activity prepared from C₃H and C57BL macrophages⁷ (Report No. 1, p.4). In addition, we have recently shown⁸ that EFs from both L. donovani and L. tropica, independent of mode of preparation, inhibit β -galactosidase from E. coli; and (b) EF is insensitive to precipitation by a wide variety of lectins^{7,9,10}. However, PNA was found by us in this work (Report No. 1, p. 4) to precipitate the EF of L. tropica after TCA-treatment, using immunodiffusion methods⁵. To further examine the interaction of EF with PNA, immunoelectrophoresis was performed. Partially purified EF from both L. donovani and L. tropica when treated with PNA ran a shorter distance on immunoelectrophoresis than did untreated EF (Report No. 1, p. 4), thus indicating formation of a soluble EF-PNA complex. In conclusion, EF possesses non-reducing galactose and even β -galactosyl residues that could serve as "handles" for affinity chromatography on galactose binding affinity columns.

Peanut lectin (PNA) was obtained from either Sigma or Makor, Jerusalem, or produced in large quantities in our laboratory by slight modification of the method of Terao et al.¹¹ (Report No. 1, p. 12). Agarose-Ricinus Communis Agglutinin 120 (1.1 mg protein per ml resin) was a product of Miles-Yeda, Rehovot. Aminoethyl cellulose was purchased from Sigma.

Isolation and phenol extraction of EF

L. tropica and *L. donovani* promastigotes were cultivated in LIT medium supplemented with 10% foetal calf serum and antibiotic -- 100 µg streptomycin and 100 u penicillin per ml. Cultures at the logarithmic phase of the growth were centrifuged 7 min at 1500 xg. The supernatant was removed and concentrated to 1/10 the original volume and dialyzed for 3 days against distilled water. The dialyzate was concentrated again and extracted with phenol. The aqueous phase was dialyzed for 3 days against distilled water and the dialyzed concentrated EF was fractionated on Sephadex G-100 column (Fig. 1)¹¹. The fractions containing EF as detected by immunodiffusion were pooled, concentrated by freeze-drying and dialyzed again for 3 days against saline. No phosphate was detected in this EF preparation¹².

Affinity chromatography using immobilized PNA

Purification of EF was achieved by binding PNA to a column of aminoethylcellulose¹³ (0.4 mg protein per ml of resin). 5 mg of EF L32 were applied to the column (12 cm long; 1 cm in diameter) and eluted with 0.02 M sodium phosphate buffer, pH 7.0 (Fig. 2). The fraction volume was 1.0 ml. Fractions were assayed for antigenic EF activity by double gel diffusion, protein¹⁴ and carbohydrate content¹⁵. EF activity eluted after the void volume. Approximately 1 mg of purified EF was obtained, representing a 3-fold purification with respect to protein and a 4-fold purification with respect to carbohydrate.

Affinity chromatography using Ricinus lectin column

In a typical experiment, 16 mg of EF were applied to a Ricinus lectin column (Agarose-Ricinus communis Agglutinin 120, 7 cm long, 1 cm in diameter). The column was first eluted with 0.02 M sodium phosphate buffer, pH 7.0 (12 fractions) followed by a similar buffer containing 50 µ M galactose (all fractions contained 2.6 ml per fraction, Fig. 3). Fractions were assayed for antigenic EF activity by double gel diffusion, protein and carbohydrate content and consequently, fractions detected by immunodiffusion pooled, dialyzed against water and lyophilized to yield purified EF (2.0 mg) possessing 8 x increase in the specific antigenic activity.

Determination of molecular weight

Partial specific volumes and molecular weights were determined by sedimentation equilibrium according to Edelstein and Schachman¹⁶ on a Beckman Model E analytical ultracentrifuge equipped with absorption optics and photoelectric scanning system. Samples (0.6 mg/ml) were dissolved in (and dialyzed against) a buffer containing 50 mM sodium phosphate, 120 mM sodium chloride and 0.02% sodium azide, pH 7.0 made with water or with deuterium oxide. Runs were conducted in 12 mm DS charcoal cells run at 20°, at 24,000 RPM in an AN-G rotor and scanning was carried out at 280 nm. Density of buffers were determined on a digital density meter, Anton Paar Model DMA 026; the buffer made with water had $\rho = 1.0067 \text{ g} \cdot \text{ml}^{-1}$ and the buffer made with D₂O had $\rho = 1.1112 \text{ g} \cdot \text{ml}^{-1}$. The partial specific volume (\bar{v}) was determined for EF L137 as $0.695 \text{ ml} \cdot \text{g}^{-1}$ and was used for this and for another EF sample (EF L52) to determine the molecular weights as 49,670 and 34,887, respectively. Samples studied were those purified on a Ricinus lectin column.

Amino acid analysis

EF L137 was analyzed following acid hydrolysis using amino acid analyzer (LKB Model 3201). See Table I for results.

Carbohydrate analysis

Monosaccharide components of EF were determined by GLC following methanolysis and trimethylsilylation [18]. See Table II for results.

Labelling expt. (purified by Ricinus).

Table I

AMINO ACID ANALYSIS OF EF PREPARATIONS

Samples examined were purified on a Ricinus lectin column

Amino acid	EF L137			EF L52		
	% of dry weight	n mole/mg	No. of residues	% of dry weight	n mole/mg	No. of residues
Lysine	2.37	162.40	8.06	0.93	63.93	2.23
Histidine	0.84	54.80	2.72	--	--	--
Arginine	1.51	86.98	4.32	0.84	48.46	1.69
Aspartic acid*	2.82	212.75	10.56	1.35	101.86	3.55
Threonine	1.94	163.76	8.13	1.33	112.13	3.91
Serine	1.56	149.07	7.40	1.55	148.00	5.16
Glutamic acid*	4.20	286.01	14.20	2.22	151.20	5.27
Proline	1.61	140.54	6.98	0.81	70.80	2.47
Glycine	1.17	156.38	7.76	0.83	111.88	3.90
Alanine	1.82	204.66	10.16	1.34	151.06	5.27
Half Cystine	0.86	71.67	23.56	1.62	135.33	4.72
Valine	1.69	145.03	7.20	--	--	--
Methionine	0.23	15.65	0.77	--	--	--
Isoleucine	0.79	60.42	3.00	0.57	43.46	1.51
Leucine	2.68	204.60	10.16	1.11	85.40	2.98
Tyrosine	3.65	201.90	10.03	3.78	209.20	7.30
Phenylalanine	2.33	141.70	7.03	2.30	139.50	4.86
Total	32.07			20.60		

*By titration¹⁷ contains 32.3 ± 3.2 acid residues per molecule.

Table II

MONOSACCHARIDE COMPOSITION OF EF PREPARATIONS

Samples examined were purified on a Ricinus lectin column.

Sugar	EF L137			EF L52		
	% of dry weight	n mole/mg	No. of residues	% of dry weight	n mole/mg	No. of residues
Arabinose	0.1	8	0.4	<0.1	-	-
Fucose	2.8	170	8.44	3.1	188	6.56
Xylose	0.4	29	1.44	0.5	32	1.12
Mannose	2.8	154	7.64	5.3	293	10.22
Galactose	11.3	628	31.19	11.6	643	22.43
Glucose	2.2	124	6.16	3	169	5.89
Galactosamine*	0.8	43	2.17	0.9	50	1.73
Total	19.6			24.5		
Total sugar ¹⁷	28			26		

*From amino acid analysis.

Sulphur analysis

EF L137 contained 1.17% and EF L52 -- 1.00% of sulphur. Subtracting the sulphur content related to cystine and methionine (Table I), the remainder (0.84% and 0.49%, respectively) could correspond to 13.11 residues of sulphate in a molecule of EF L137 and 5.57 residues of sulphate in a molecule of EF L52.

Water determination

A sample of EF L137 (10.3 mg) was checked for the water content by dissolving in D₂O (0.5 ml); 16 h later the water resonance was measured and integrated (¹H NMR, Varian model FT 80A). Calibration for water content was performed by the addition of 5 and 10 μ l of water to the EF solution followed by repeated measurements. Water present in D₂O and the theoretical amount of water obtainable through the exchange of amino acid and sugar residues of EF with D₂O were subtracted from the observed value. The corrected value of water content in EF L137 was determined as 35.6% (19.8 μ mole/mg).

Taking advantage of the binding of Leishmanial secreted factors (EFs) to galactose binding lectins, purification of these compounds was achieved on peanut lectin and Ricinus lectin columns (Figs. 2 and 3). Consequently, structured studies of purified EFs were carried out: molecular weight was determined by sedimentation equilibrium as 49,670 for EF L137 and 34,887 for EF L52. EF samples were composed of 32-20% amino acids, 20-25% carbohydrates and a large proportion of the rest was water (EF L137, 35.6%).

The amino acid composition was low in sulphur containing amino acids and high in acidic amino acids. Some of these carboxylic acid residues could be involved in amide bond formation, a proportion of which might be present at the linkage to the carbohydrate moiety, while the rest alongside with apparently present sulphate residues could be responsible for the established acidic (32.3 acid residues per molecule, Table I) (polyanionic) properties (4,7) of EF.

The carbohydrate portion of EF (Table I) is particularly rich in galactose, a fraction of which must be at the nonreducing ends, and contains also mannose, glucose, fucose and a small proportion of xylose and Arabinose.

In the process of affinity chromatography, we have observed (Report No.2, p.6) microheterogeneity, demonstrated, for instance, by the increase of galactose/mannose ratio if EF peaks between early and late fractions can be understood in view of the galactose binding properties of the Ricinus lectin column.

An alternative system of affinity chromatography, affinity chromatography using antibody bound to Sepharose, was also investigated (Report No.1, p.6) but requires further refinement.

3. Radiolimmunoassay (RIA) for the Diagnosis of Leishmaniasis:

RIA technique, based on the binding of antibody to solid phase coating EF, followed by protein A labeling, was used as previously described by Avraham et al (19). The test was done in both polystyrene tubes (100mm x 13mm, Nunc, Denmark) and 96 wells microplates (Sterilin, England). Coating with EF was done in tubes and microplates either treated with glutaraldehyde or left untreated. EFs and glutaraldehyde were diluted in borate buffer saline (BBS) pH 8 and dilutions of sera and protein A were made in BBS containing 10% foetal calf serum. The assay for both L. tropica and L. donovani EF was as follows: 100 µl ¹²⁵I-protein A providing about 50,000 counts/min were added. The reaction was terminated by the addition of 120 µl of 0.1N NaOH. The solution from each well was then transferred to plastic tubes and the radioactivity measured.

For inhibition studies, 0.2 ml of 1:10 dilutions of antisera were mixed with serial dilutions of EF at a final volume of 0.2ml. After incubation for 30 min at 37°C, the adsorbed sera were added to the EF coated microplates and the radioactivity was measured.

Results obtained by this method are as follows:

a. Fractionation of dissociated EF-antibody complexes

When dissociated complex of *L. donovani* EF and rabbit anti-EF antibodies were separated on a Sephadex G-100 column, two main fractions were obtained; firstly, the antibody fraction (MW=178,000), followed by an EF fraction (MW=33,000) (Fig. 4). After dialysis, both fractions displayed the characteristic behavior of the original starting material. The antibody fraction displayed only one band after immunoelectrophoresis against goat anti-rabbit serum (Fig. 5). Similarly, the EF fraction showed one precipitating band, which ran to the same locus as the original untreated EF. This technique, used alone or in combination with one of the previously described methods, leads to greater purification of EF as well as to the separation of monospecific anti-EF rabbit IgG.

b. Coating the plate

Under the conditions described, EF that had been purified by extraction with phenol, followed by gel filtration on Sephadex G-100, was found to be the only EF preparation that could be bound to tubes and microplate wells. All the other EF preparations obtained by either precipitation and chromatography or by the dissociation of immune complexes, did not bind to the plates and showed the same activity with antibody as untreated control EF. It appears that the absolute amount of EF coupled to the plate depends essentially on the level of purification and the nature of the medium component that acts as a carrier, rather than the amount of antigenic determinants of the EF.

The concentration of EF used for coating the wells was 0.6 to 1.2 mg/ml. Immunodiffusion of this EF with homologous antiserum produced an immune precipitate at a maximum dilution 1:2. Concentration exceeding 1.2 mg/ml increased the strength of the binding without affecting the specificity (Fig. 6). At concentrations less than 0.35 mg/ml, no antibodies were detected. Almost no differences in extinction values were seen with wells coated or uncoated with glutaraldehyde.

c. Specificity of the assay

The interaction of antisera with different EFs and the specificity of the reaction is given in Fig. 7. The results obtained indicated that anti-*L. tropica* and anti-*L. donovani* reacted only with homologous EF. Owing to the higher concentration of rabbit anti-*L. donovani* IgG, as measured by immunodiffusion, higher binding was observed with this antiserum, as compared with anti-*L. tropica* serum at corresponding dilutions.

In most cases, the activity obtained with the heterologous antiserum was almost the same as that of control normal rabbit serum. With antisera diluted at 1:10, 18% of the anti-L. donovani antibodies were bound to L. tropica EF, 32% of anti-L. tropica antibodies were bound to L. donovani EF and 12.5% to 16% of normal rabbit antibodies were bound nonspecifically to L. donovani and L. tropica EF respectively.

By adding monospecific anti-L. donovani EF IgG to plates coated with monologous EF (1.2 mg/ml), it was found that as little as 0.06-0.12 µg/ml of anti EF IgG could be detected. Based on the results with the monospecific IgG, the amount of anti-L. donovani EF in whole rabbit anti-L. donovani serum was 0.06 mg/ml.

d. Inhibition study

As indicated in Fig. 8, the reaction of both anti-L. donovani and anti-L. tropica sera could be completely inhibited by preincubation with homologous EF. 300 µg of L. donovani and L. tropica EF were sufficient to block all the anti-EF antibodies present in 1 ml of homologous antiserum, and as little as 10-20 µg/ml of EF could be detected.

e. Detection of anti-EF antibodies in mice and humans suffering from cutaneous leishmaniasis

Anti-EF activity in sera from humans infected with L. tropica is shown in Table III. Of 7 sera examined from patients with active or a history of cutaneous leishmaniasis, 4 (57%) with parasites present in their skin lesion showed maximum activity of 1.4 to 2.25 times higher than normal uninfected control. No anti-EF antibodies were detected in the serum of a patient with active Leishmaniasis recidiva.

Serum collected from BALB/c mice 40 days after infection with L. tropica, was also assayed for anti-EF antibodies. These mice showed well-developed lesions and parasites were detected in both their skin lesion and in their spleens. The anti-EF antibody activity detected in these sera was as high as 25.6 times that of a normal non-infected mouse serum.

The immune response to leishmanial infection is considered to be mainly cell mediated (20); however, the production of non-protective antibodies has been detected in all forms of leishmaniasis, i.e. cutaneous, mucocutaneous and visceral leishmaniasis (21-27). The procedures for measuring these antibodies have not proved totally satisfactory in the diagnosis of leishmaniasis, owing to the limited sensitivity of the tests used, especially in cutaneous leishmaniasis, and because of cross-reactivity between different species of Leishmania and other microorganisms. While the antibody titers in

Table III. Anti-L. tropica EF antibody activity in sera from patients with simple cutaneous leishmaniasis caused by L. tropica major. Serum E is from a patient with leishmaniasis recidiva caused by L. tropica minor. The numbers in brackets are the results compared with the activity measured in normal human serum. The control is the mean of 7 normal sera.

		serum	serum	serum	serum	serum	serum	serum	serum	control
		A	B	C	D	E	F	G		serum
Number of lesions		24	45	20	6	1	6	8		
microscopic examination	smear	+	+	+	+	+	-	-		
	culture	+	+	+	+	+	-	-		
serum dilution					% Binding					
1:5	16.90(2.60)	6.86(1.06)	8.90(1.36)	5.43(0.83)	9.14(1.40)	8.54(1.31)	5.43(0.83)	6.50		
1:10	9.10(1.87)	5.99(1.23)	6.59(1.35)	3.95(0.10)	7.58(1.56)	5.67(1.16)	3.71(0.76)	4.86		
1:25	5.19(1.78)	3.59(1.23)	4.27(1.47)	2.19(0.75)	4.47(1.54)	2.63(0.90)	2.47(0.85)	2.90		
1:50	3.31(1.46)	3.19(1.41)	2.11(0.93)	1.55(10.68)	2.67(1.18)	1.55(0.70)	1.75(0.77)	2.26		
1:100	2.27(1.50)	1.87(1.23)	1.34(0.98)	1.11(0.73)	1.83(1.21)	1.03(0.68)	1.35(0.89)	1.51		

visceral leishmaniasis in man are high and rise sharply during infection (25,28), in patients with uncomplicated self-curing cutaneous leishmaniasis, the titers are low to negligible (22,24,27). In mucocutaneous leishmaniasis, antibody is only detected when metastases occur (22,27,28). Furthermore, the fluorescent antibody test (FAT) used in mucocutaneous leishmaniasis is only genus specific (29) and non-specific cross-reactions occur with other genera, e.g., *Trypanosoma cruzi* (29). Similarly, serum from patients with visceral leishmaniasis may cross-react with *T. cruzi* and *Mycobacteria* in both the FAT and the Indirect hemagglutination (IHA) tests (30,31). Allain and Kagan (32) in a well-controlled study, also indicated the high cross-reactivity obtained with three strains of *Leishmania* in the direct agglutination test. In this study, where enzyme-treated, formalin-fixed promastigotes were used, 61-96% of the sera from patients with kala-azar also reacted with *L. tropica* and *L. braziliensis* parasites. In addition, sera from patients with American cutaneous leishmaniasis were 54 to 55% positive with the other two strains.

These problems appear to be a feature of the leishmaniasis, the diagnostic tests and of the antigens used. In these tests, and the ELISA in which the sensitivity of the test is greatly increased (33,34), intact parasites or somatic soluble antigens were used. This may account for the non-specificity and cross-reactivity with other microorganisms.

Unlike somatic antigens, leishmanial EF is antigenically specific for leishmanial serotypic groups. In a previous paper (35), we used EF in coagglutination and indirect hemagglutination tests. In both types of test, the reaction with EF was sensitive and specific.

The immunological specificity of leishmanial EFs and their isolation from other leishmanial antigens and contaminating medium components (4,6) was utilized in this study to provide a non-sensitive means of detecting and measuring the antibody response to leishmanial infection. Since EF is haptenic (4), anti-leishmanial EF antibodies are produced in rabbits by the inoculation of whole promastigotes (1). Although the antibodies obtained by this procedure bind with EF, some also bind with somatic components of the parasites.

The data presented in this study indicate that the binding of EF to antibody is sufficiently avid for this to be used as a basis for RIA, and this is perfectly adequate for the measurement of both EF and antibody titers. Three further features were found to be of potential value for this test. Firstly, it was found that it is not essential to have highly purified EF for immobilization on the solid phase, and, when impure EF was used, sensitivity was not affected. Secondly, the use of isolated leishmanial antigen avoids cross-reactivity that occurs with other procedures. Finally, there is no limitation as to the species of origin of the antibodies. Furthermore, while the method of purification was significant in the binding

of EF to the plates, no effect was observed with regard to inhibition study. The EFs produced by the three methods described: phenol extraction, precipitation and chromatography (4) and dissociating antigen-antibody complex, similarly inhibited the activity of homologous antibody, when used at the same concentration.

By using homologous and heterologous antisera, the specificity of the test was found to be high in all instances tested. Furthermore, by inhibition studies on antibodies against *L. tropica* and *L. donovani*, EF was demonstrated only after absorption with heterologous EF, but not after absorption with homologous EF. Thus, antibodies were neutralized only with homologous EF.

In a comparative study, Rassam & Al-Mudhaffar (36) indicated the superiority of the micro ELISA as compared to gel diffusion immunoelectrophoresis and counter current electrophoreses for the diagnosis of visceral leishmaniasis. 1.5 µg of protein antigen and 30 µl of serum were adequate for the test. Similarly, in our work (37), we sensitized papain-treated red blood cells with EF, at a concentration of 15 to 30 µg/ml, and in coagglutination test purified *L. donovani* yields positive reactions at a concentration of 7.8 µg/ml. In the present work we found that 1-2 µl of antiserum are sufficient for the RIA, and as little as 0.6-1.2 µg/ml anti-EF IgG or 1-2 µg/ml EF could be detected.

Based on the results obtained with sera from humans and mice infected with *L. tropica*, it seems that the RIA developed in this study will be of value in both immunological and diagnostic studies of leishmaniasis. When sera from patients with cutaneous leishmaniasis were tested, antibodies could be detected at a maximum level of 2.25 times higher than normal controls. However, in two cases with active cutaneous leishmaniasis, antibody levels were the same as that of normal controls. In sera from mice infected with the same type of parasite, but where the disease became visceral, the titers were 25.6 times higher than normal controls. These results are not surprising, since, in cutaneous leishmaniasis, antibodies may only be detected when the lymphatics are involved (24,25); whereas in visceral leishmaniasis, the IgG titers are greatly elevated (24,27). This illustrates the potential of this RIA technique in the diagnosis of visceral and mucocutaneous leishmaniasis.

In conclusion, we believe that the use of leishmanial EF for RIA will prove valuable in the diagnosis of leishmaniasis. RIA is generally a very sensitive test. The use of EF has the advantage of its being stable and specific in this test. Undoubtedly, experience will lead to modification and refinement of the technique, depending on its specific application. However, sufficient details have been presented here to enable its immediate use with regard to the detection of cutaneous leishmaniasis and the measurement of EF levels.

4. Surface Carbohydrate (Related to EF) and the Binding of Leishmania Promastigotes to Macrophages

It is known that liver and peritoneal macrophages have specific galactose-binding receptors (hepatic binding protein, HBP) which are able to bind neuraminidase-treated cells where galactose is the non-reducing sugar as a prelude to their catabolism (36-39). It was demonstrated recently, however, that the galactose or the N-acetyl-galactosamine specificity of HBP is not high and additional mono-saccharides (e.g. glucose) can compete, though less favourably, for HBP binding (36).

The first annual report, section 5b, has described the presence of a high galactose content in EF, a finding that was further supported by our current analyses, p. 8. In addition, we have demonstrated that promastigotes of both *L. tropica* and *L. donovani* are agglutinated by low concentrations of peanut lectin and *Ricinus* lectin and that dissociation is readily achieved by the addition of galactose. This indicates the presence of oligosaccharides possessing terminal non-reducing galactose as a partial structure of the promastigote surface (first annual report section 2a and current results concerning *Ricinus* lectin).

We propose that the host cell (macrophage) membrane might contain a galactose-binding receptor, possibly the well-documented hepatic binding protein (HBP), capable of binding either the parasite or EF. *Leishmania* may thus be attached initially to the macrophage via such a galactose-binding site and be subsequently engulfed by the macrophage.

For attachment study of promastigotes to macrophages, the culture system of Handman & Spira (40) was adapted. Peritoneal exudate cells (PEC), mostly macrophages, from C3H mice were harvested 5 days after I.P. stimulation with 2 ml thioglycolate. On the day of harvest, the cells were collected in McCoy's medium containing 5 units of heparin, 100 µg streptomycin and 100 units penicillin per ml. The cell concentration was brought to 5×10^5 cells/ml and 1 ml was plated into each well of the 24 wells microplate. Before adding the PEC, 12 mm diameter sterile coverslips were placed in each well. 24 hours after incubation at 37°C in an atmosphere of 5% CO₂ in air, the medium was changed, removed, and 200 µl of the sugar in the desired concentration made in Hank's balanced salt solution containing 1% albumin (HBSSA) was added. After 15 min at 37°C, the sugar was replaced with 400 µl of the same sugar, at the same concentration containing 2×10^7 washed promastigotes. After a further 10 min at 37°C, the PEC were washed 5 times with phosphate buffer, fixed and stained with Giemsa. Following counting, the parasite attachment index (PAI), (section 8) was calculated.

Figure 9 shows that different sugars (at 0.3 - 0.5 M) inhibit the attachment of promastigotes to macrophages. Lactose, Gal- β (1 \rightarrow 4)Glc, being the most efficient. Lower concentrations of sugars promote attachment while 1 M and higher concentrations are toxic to host (macrophage) cells. Apart from standard error possible (Ca. 10%), the effect of lower saccharide concentrations may be due to additional sugar nutrient. Sugars at concentrations of 0.5 M are non-toxic to both promastigotes and macrophages. Following the addition of 0.5 M saccharide and incubation as described, the cells are washed and give normal infective rates.

5. Conclusions

In the vertebrate host, *Leishmania* are obligatory intracellular parasites of the mononuclear phagocyte, in which they grow and multiply as amastigotes even in the presence of lysosomal enzymes (41,42). The mechanism of survival of these microorganisms within these cells is still unknown. It was recently shown that EF acts as a conditioner for amastigotes in macrophages from resistant animals (40).

- a. EF, previously purified by physical and chemical methods was further purified here by affinity chromatography on peanut and Ricinus lectin columns. The purified samples were characterized and analysed. The results show a notable proportion of galactose in EF and clarify the reasons for the polyanionic properties of EF. The sugar component is of particular importance since it carries the antigenic determinants of *Leishmania* [2,7], and is relevant to the infective process of this parasite (7,43).
- b. A radioimmunoassay for the quantitative determination of anti-leishmanial excreted factor (EF) antibody in rabbit sera was developed. The assay, using *Leishmania tropica* and *Leishmania donovani* promastigote EF, purified by extraction with phenol followed by Sephadex G-100 column, was shown to be sensitive and reproducible, and may be used for the diagnosis of leishmaniasis in man and animals. Using monospecific anti-EF antibodies, titers as low as 0.6-1.2 μ g/ml of anti-EF IgG could be detected. The specificity of the assay was assessed by inhibition with homologous and heterologous EF. Only minor cross-reactivity with heterologous EF was observed. Sera from human subjects and mice infected with *L. tropica*, showed 2.60 and 25.6 times more anti-EF activity, as compared with non-infected controls.
- c. *Leishmania tropica* promastigotes are easily attached to and engulfed by C₃H peritoneal macrophages *in vitro* at 37°C. Different sugars at 0.3-0.5 M inhibited *in vitro* the attachment of *L. tropica* promastigotes to C₃H peritoneal macrophages with Lactose (Gal- β (1 \rightarrow 4)Glc) being the most efficient. Other sugars which were less inhibitory but still were found to be effective

were: raffinose>methyl α -D-mannopyranoside>methyl β -D-galactopyranoside>methyl α -D-galactopyranoside>D-arabinose>D-glucose. Sugars at concentrations of 0.5 M are non-toxic to both promastigotes and macrophages, while 1 M and higher concentrations are toxic to host cells.

This study suggests that Leishmania, an obligatory intracellular parasite, uses as a means of entering the host cell a cellular mechanism similar to that which is used in the removal of damaged cells from blood circulation. Once the parasite is inside the cells, the EF it produces might have a protective function, being inhibitory to some of the host cell lysosomal enzymes.

Fig.1 Elution profile of crude *L.tropica* EF treated with phenol and put through a Sephadex G-100 column.
A 5ml sample was fractionated on a 2.5x40 cm column of Sephadex G-100, with a flow rate of 20ml per hr, using distilled water as the eluent.

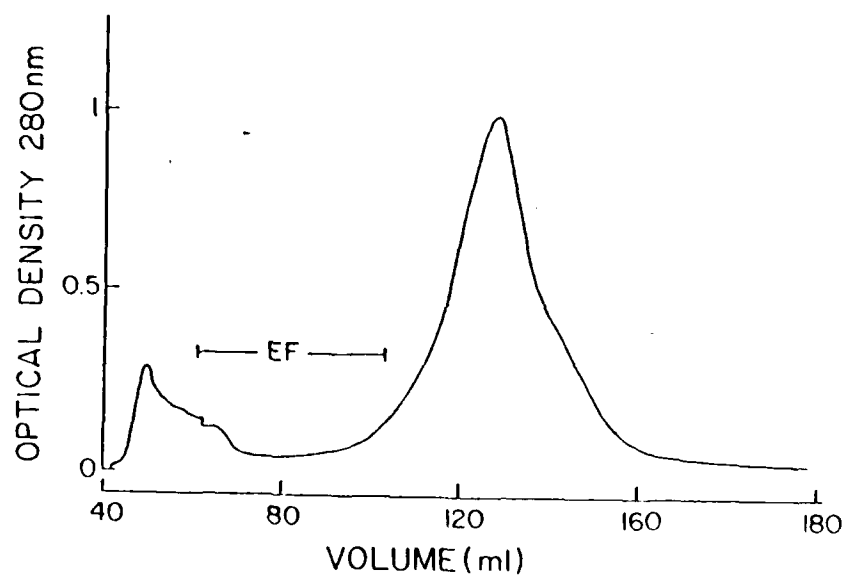


Fig. 2 Affinity chromatography of EF L32 on a column of immobilized peanut lectin. ●---●, protein ($\mu\text{g}/\text{fraction}$); △---△, carbohydrate ($\mu\text{g}/\text{fraction}$); x---x, EF activity.

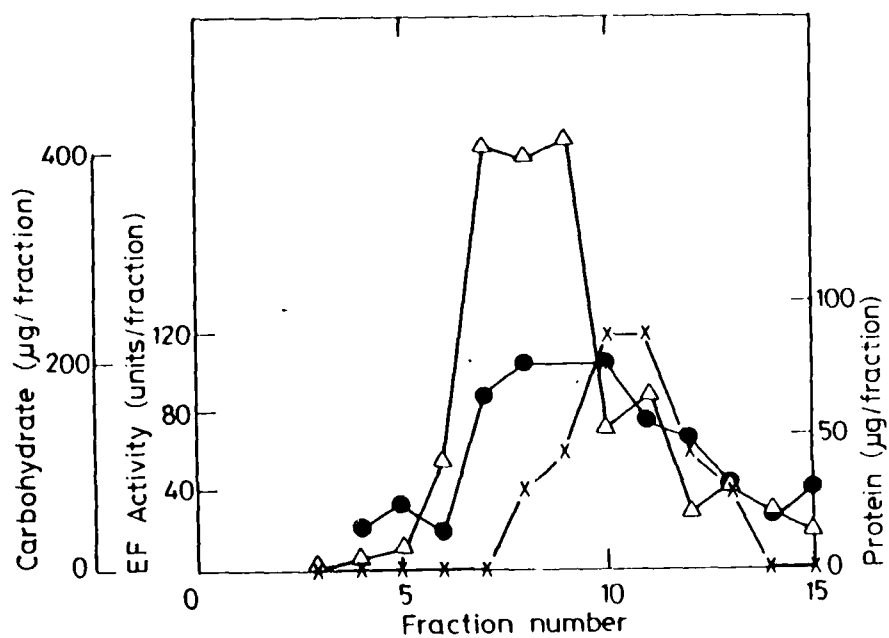


Fig. 3 Affinity chromatography of EF L137 on a column of immobilized Ricinus lectin. ●---●, protein ($\mu\text{g}/\text{fraction}$); Δ --- Δ , carbohydrate ($\mu\text{g}/\text{fraction}$); x---x, EF activity. Indicated fractions were pooled.

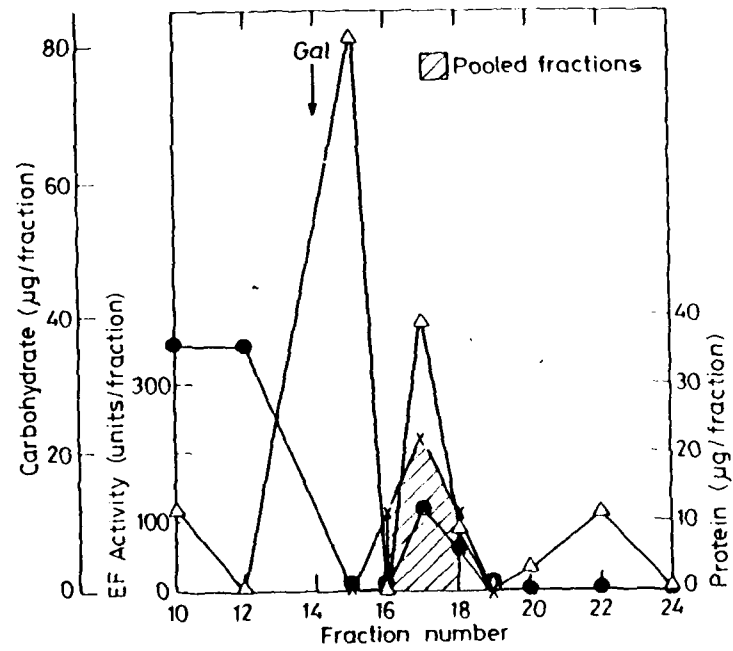


Fig. 4 Separation of *Leishmania donovani* EF and rabbit anti *L. donovani* IgG from dissociated immune complexes on Sephadex G-100 column. A 2 ml sample was fractionated on a 1.5 x 79 cm column of Sephadex G-100, with a flow rate of 43 ml per hr, using 2 M Sodium thiocyanate as eluting buffer.

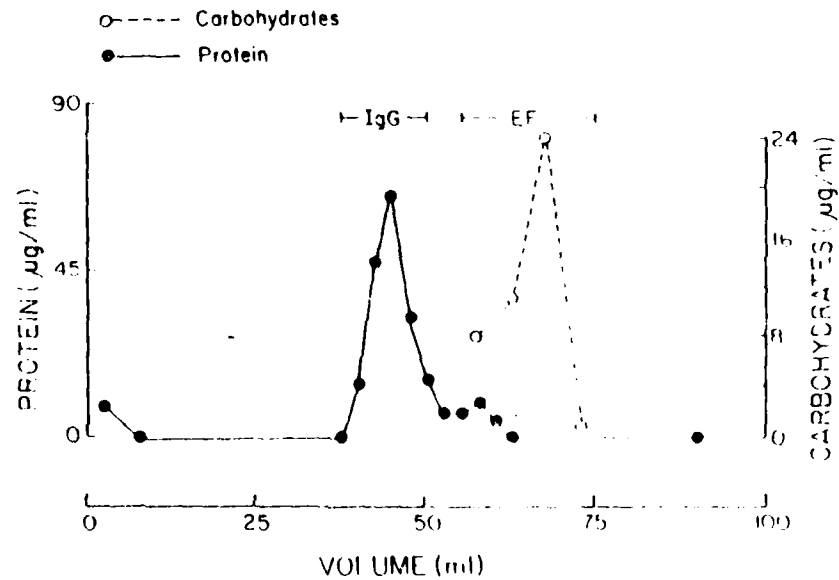


Fig. 5 Immunelectrophoresis of *L. donovani* EF and immune rabbit IgG obtained from dissociated immune complexes (1) crude EF (2) dissociated EF (3) dissociated IgG (4) normal rabbit serum. The upper trough contains rabbit anti-*L. donovani* antiserum, and the lower trough contains goat anti-rabbit serum.

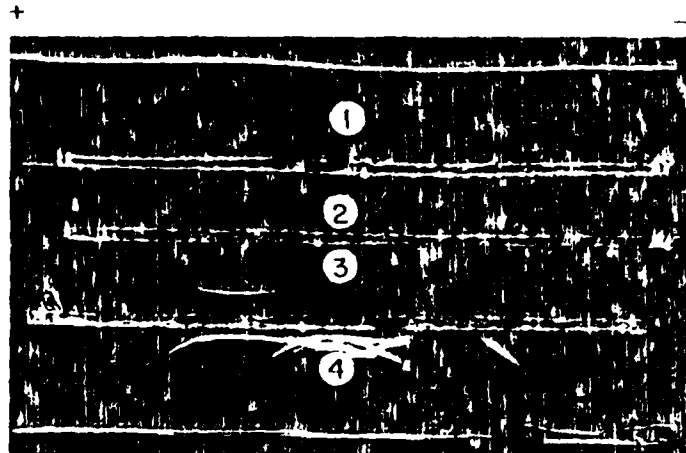


Fig. 6 The effect of different concentrations of coating *L. tropica* EF on the binding of antibodies from homologous antiserum at dilution 1:25. The EF showed a precipitation band with whole antiserum at 1000 $\mu\text{g/ml}$.

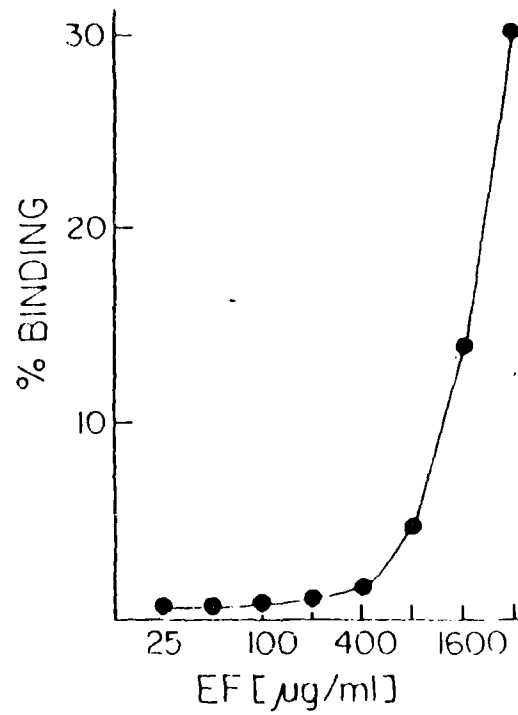


Fig. 7 Binding of rabbit anti-*L. tropica* antiserum (), rabbit anti-*L. donovani* antiserum (▨) and normal rabbit serum (■) to plates coated with *L. donovani* or *L. tropica* EF.

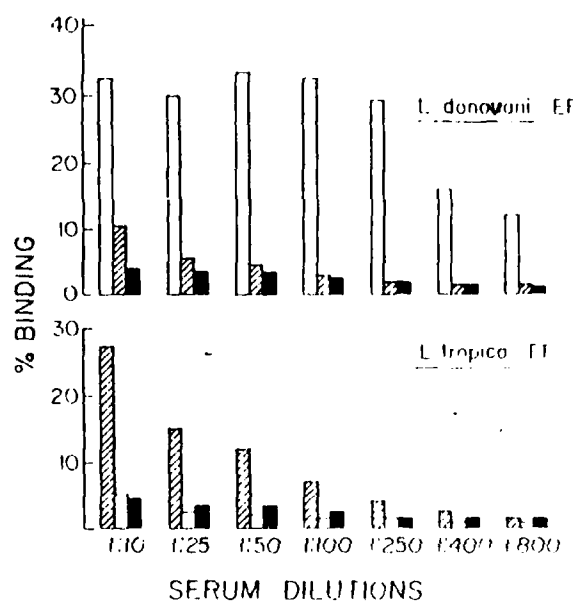


Fig. 8 Effect of absorption of anti-*L. tropica* and anti-*L. donovani* antiserum with homologous and heterologous EF. Anti-*L. tropica* absorbed to homologous EF (●); anti-*L. donovani* absorbed to homologous EF (▲). The inhibition is calculated as follows: $100(1-xy^{-1})$, where x equals the residual antiserum activity after adsorption with homologous EF and y the residual activity of the same antiserum after adsorption with heterologous EF.

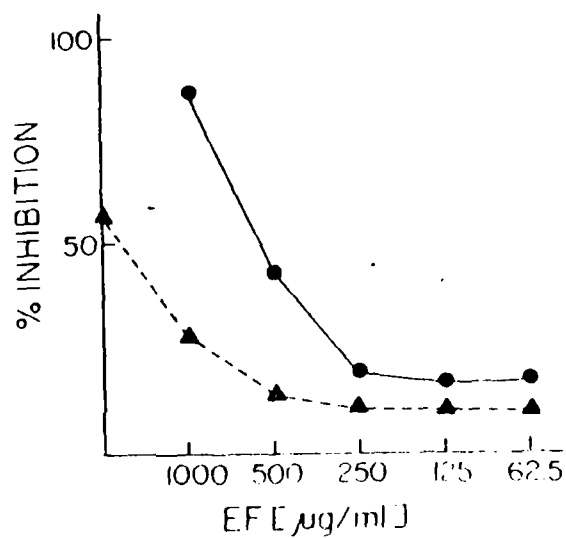
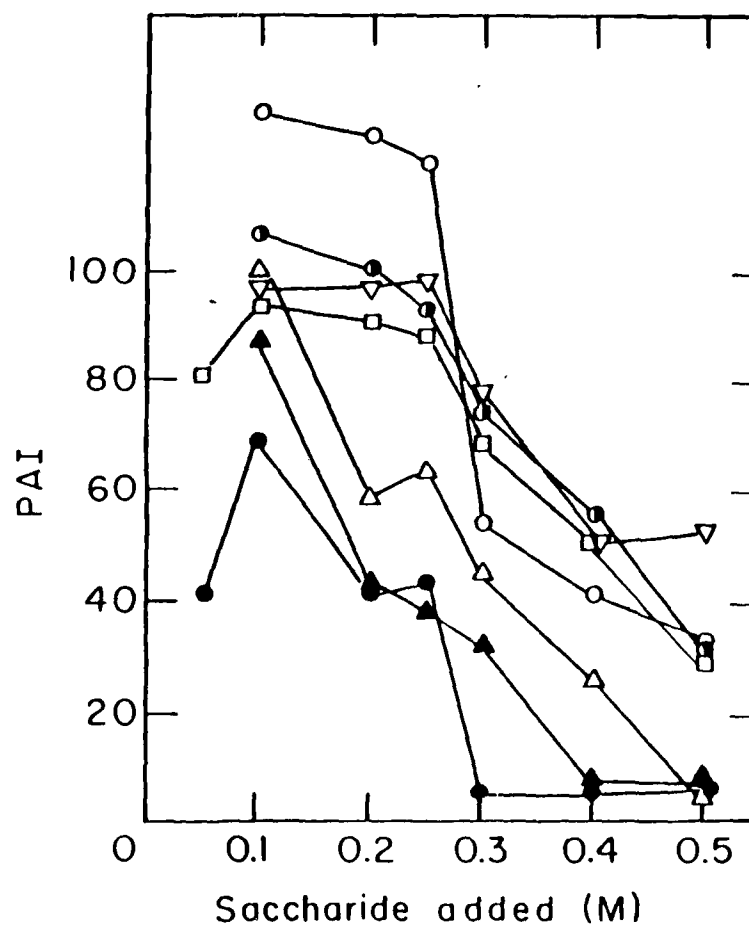


Fig. 9 The effect of sugars at different concentrations on the parasite attachment index (PAI) of *L. tropica* (L 137) to C₃H mouse macrophages.
 ● - Lactose; ○ - Glucose; ◐ - Methyl α -D-galactopyranoside;
 ◑ - Methyl β -D-galactopyranoside; ▲ - Raffinose;
 △ - Methyl- α -D-mannopyranoside; ▽ - D-arabinose.



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8. Glossary

EF Factor excreted by Leishmania (Excreted Factor). EF preparations are designated with digits indicating the leishmanial source.

Leishmanial strains

L137 Leishmania tropica LRC L137

L52 Leishmania donovani LRC L52

These strains were obtained from the WHO Leishmania Reference Centre collection maintained in the Department of Protozoology in Jerusalem.

PNA peanut lectin

RIA radioimmunoassay

PAI parasite attachment index =

$$\frac{a_{\text{exp}} \times b_{\text{exp}} \times 100}{a_{\text{cont}} \times b_{\text{cont}}}$$

a = percent of host cells where attachment is apparent. 400 host cells were counted.

b = average no. of attached promastigotes per cell. 100 cells with attached promastigotes were counted.

9. Publications Supported by This Contract

1. El-On, J.; Zehavi, U.; Slutzky, G.M.; Abrahams, J.C. and Greenblatt, C.L. 1980. A possible role for leishmanial excreted factor (EF) in the infective process. The Biochemistry of Parasites, Satellite Meeting of the 13th FEBS Meeting, Jerusalem, Israel.
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